Imetelstat, a Telomerase Inhibitor, Is Capable of Depleting Myelofibrosis Stem and Progenitor Cells

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Supplemental Methods

Western blotting

Primary CB and splenic MF $CD34^+$ cells were lysed. Protein lysates were then analyzed by 4-20% SDS-PAGE as previously described. Rabbit anti-hTERT, and corresponding HRP-conjugated secondary antibodies were procured from Cell Signaling Technology, Inc., (Danvers, MA). The proteins were visualized by enhanced chemiluminescence detection (ECL, Amersham Pharmacia Biotech, Piscataway, NJ).

Telomerase activity (TA) assays

A quantitative telomerase detection kit (QDT, Allied Biotech, Inc., Benicia, CA) was utilized to measure TA according to the manufacturer's instructions. The TA was measured in 0.01-0.1μg cell lysates by monitoring telomeric repeat synthesis in the presence of a telomere-specific sequence oligonucleotide substrate. The newly synthesized DNA was then detected by PCR. The results were plotted as the number of real-time PCR threshold cycles (C_T) required for detection of SYBR green fluorescence resulting from binding to the resultant PCR product. Heat-inactivated telomerase was used as a negative control for cell extracts with each experimental condition. Increased TA was associated with increased double stranded DNA synthesis which required fewer PCR cycles (C_T) (e.g. the lower the C_T number, the higher the TA).

Telomere length analysis

For analysis of telomere length, a flow-fluorescence in situ hybridization (Flow-FISH) was performed with a Telomere PNA Kit/FITC for Flow Cytometry (Agilent, Santa Clara, CA). Equal number of primary MF or normal CB $CD34^+$ cells were re-suspended in micro-centrifuge tubes either in the presence of hybridization solution without FITC-conjugated peptide nucleic acid (PNA) telomere probe or in hybridization solution containing the probe. The tubes were placed in a pre-warmed heating block adjusted to 82 °C for 10 mins allowing for the sample DNA to be denatured. The tubes were then placed in the dark at room temperature (RT) overnight for the probe to hybridize with TTAGGG telomere repeats. The hybridization was followed by two 10-minute post-hybridization washes with a wash solution at 40 °C. After the last wash step, cells were stained with CD34 and CD38 mAb and incubated with the DNA solution for 2-3 hours prior to flow cytometric analysis. Telomere fluorescence intensity (TFI) of $CD34^+$, $CD34^+CD38^-$ and $CD34^+CD38^+$ cells from each sample were calculated as following: TFI= Mean fluorescence intensity (MFI) of FITC-PNA with probe-MFI of FITC-PNA without probe. The higher TFI, the longer telomere.

Supplemental Table

Table S1 Clinical Characteristics of MF Patients Studied

Patient #	Gender	Age	Diagnosis	JAK2V617F Allele Burden (%)*	CALR Status*	MPL mutation*
1	F	70	PV- MF	85.0	N/A	WT
2	M	64	PMF	0	WT	WT
3	M	79	PMF	2.4	WT	WT
4	M	67	PMF	0	46-bp Deletion	WT
5	F	45	PV- MF	90.0	WT	WT
6	F	64	PV- MF	78.0	WT	WT
7	M	76	PMF	24.9	WT	WT
8	M	73	PMF	85.1	WT	WT
9	F	52	ET-MF	0	Deletion	WT
10	M	58	PV-MF	0	WT	N/A
11	F	68	PMF	0	N/A	WT
12	F	65	PMF	49.5	N/A	WT
13	F	76	PMF	46.5	N/A	N/A
14	F	68	PMF	0	N/A	WT
15	M	72	PV- MF	0	N/A	WT
16	M	66	PMF	0	WT	WT
17	F	73	ET-MF	0.3	WT	WT
18	M	51	PMF	28.0	N/A	WT

* The *JAK2V617F* status of each MF patient was determined by analyzing PB granulocytes utilizing real-time allele-specific polymerase chain reaction (AS-PCR) assay. Mutational analysis of *CALR* was performed by sequencing regions of DNA where known mutations in *CALR* have been previously described. *MPLW515L/K* mutations were detected by AS-PCR. Pt 1-10,16-18: spleen sample; Pt11-15: PB sample.

WT: Wild type. N/A: Not Available.

Supplemental Figures

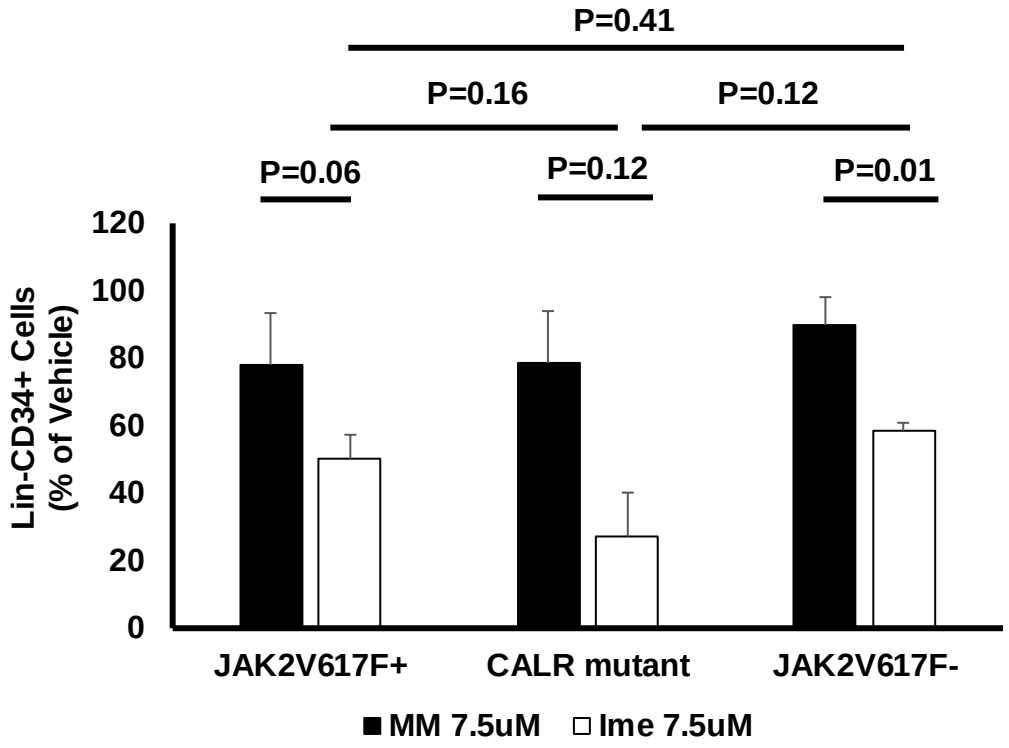

Figure S1: Inhibitory effects of imetelstat on MF CD34$^+$ cells are independent of *JAK2V617F* or *CALR* mutational status. CD34$^+$ cells from MF patients having JAK2V617F (n=8), carrying a CALR mutation (Pt 4 and Pt 9), or lacking JAK2V617F (n=4) were treated with imetelstat, MM (7.5μM) or vehicle alone as described in the Materials and Methods. Seven days after the treatment, cells were enumerated and analyzed with antibody staining and flow cytometry.

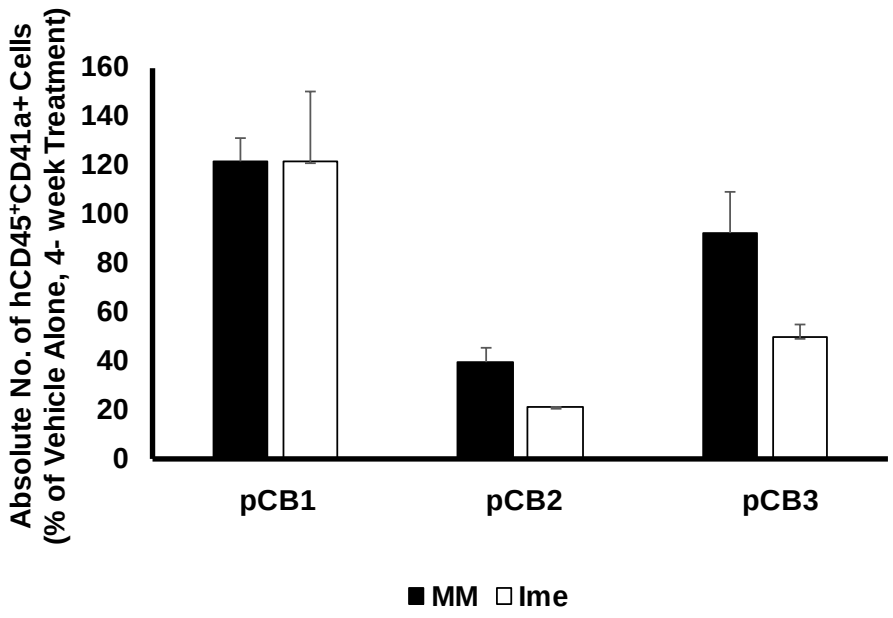

Figure S2: Effect of imetelstat treatment on normal megakaryogenesis. NSG mice were transplanted with CB $CD34^+$ cells and were treated with vehicle alone, MM or Ime (30mg/kg) for 4 weeks. 4 months after the transplantation (3 months after the discontinuation of the drug treatment), the absolute number of $hCD45^+CD41a^+$ cells present in the marrow of recipient mice was calculated as described in the Materials and Methods. The absolute number of $hCD45^+CD41a^+$ megakaryocytes in the mice receiving 30mg/kg imetelstat was reduced in 2 of the 3 pooled normal $CD34^+$ cell samples transplanted as compared with the number of cells present in mice receiving vehicle alone and same dose of MM. The data was presented as the percentage of the absolute number of $hCD45^+CD41a^+$ cells present in the marrow of recipient mice treated with MM or Ime relative to that detected in the mice treated with vehicle alone. P=0.63, MM vs. Ime (3 samples). pCB: Pooled cord blood $CD34^+$ cells.

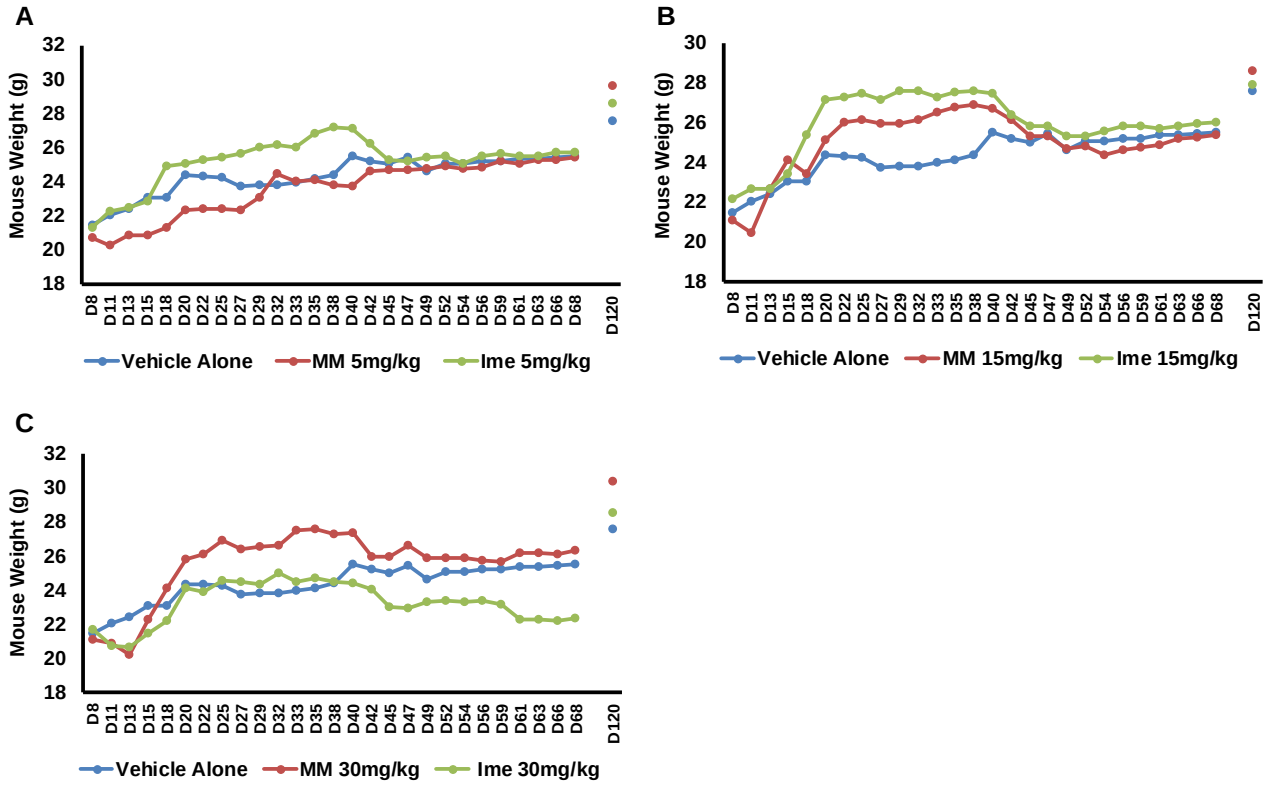

Figure S3: Body weight of NSG mice transplanted with normal cord blood CD34$^+$ cells during the treatment with different drugs and at the time the mice were sacrificed. (A-C) On Day 8 (one week after the transplantation), the mice started receiving vehicle alone, mismatched oligonucleotides (MM, 5-30mg/kg) or imetelstat (Ime, 5-30mg/kg) intraperitoneally (IP) thrice weekly for 4-8 weeks and the mice were sacrifice 4 months (D120) after the transplantation. Representative results are shown with pCB3 (pooled cord blood 3) CD34$^+$ cells and treatment for 8 weeks. The treatment with lower doses (A: 5mg/kg; B:15mg/kg) of either MM or imetelstat had no effect on mouse body weight, the higher doses of imetelstat (C: 30mg/kg), however, led to a reduction in body weight which started as early as 3 days after the treatment. X-axis indicates days after the transplantation.

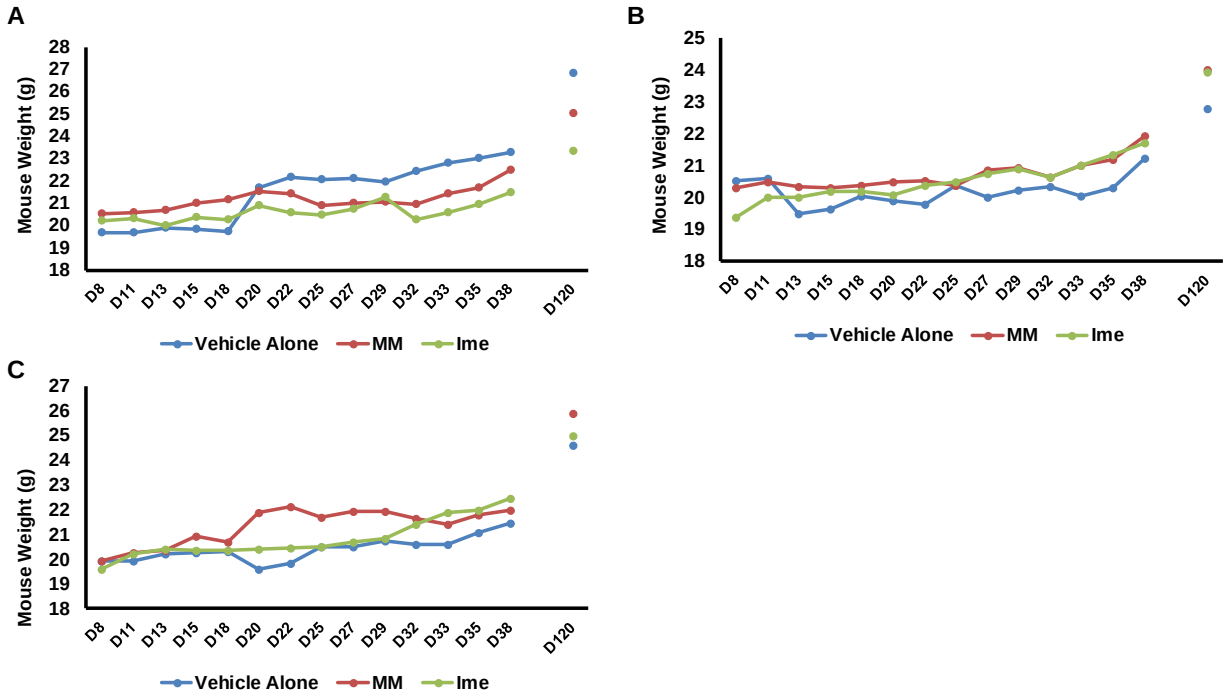

Figure S4: Body weight of NSG mice transplanted with splenic MF CD34$^+$ cells during the treatment with different drugs and at the time the mice were sacrificed. (A-C) NSG mice were transplanted with splenic CD34$^+$ cells from Pt 5 (A), Pt 8 (B) or Pt 10 (C). One week after the transplantation (Day 8), the mice started being treated with vehicle alone, mismatched oligonucleotides (MM, 15mg/kg) or imetelstat (Ime, 15mg/kg) intraperitoneally (IP) thrice weekly for 4 weeks and the mice were sacrifice 4 months (D120) after the transplantation. The treatment with imetelstat resulted in a modest reduction (3.2-9.8%) in body weight of mice transplanted with Pt 5 samples (A) starting 12 days after the treatment but didn't affect the body weight of mice transplanted with samples from the other 2 patients (B-C). X-axis indicates days after the transplantation.

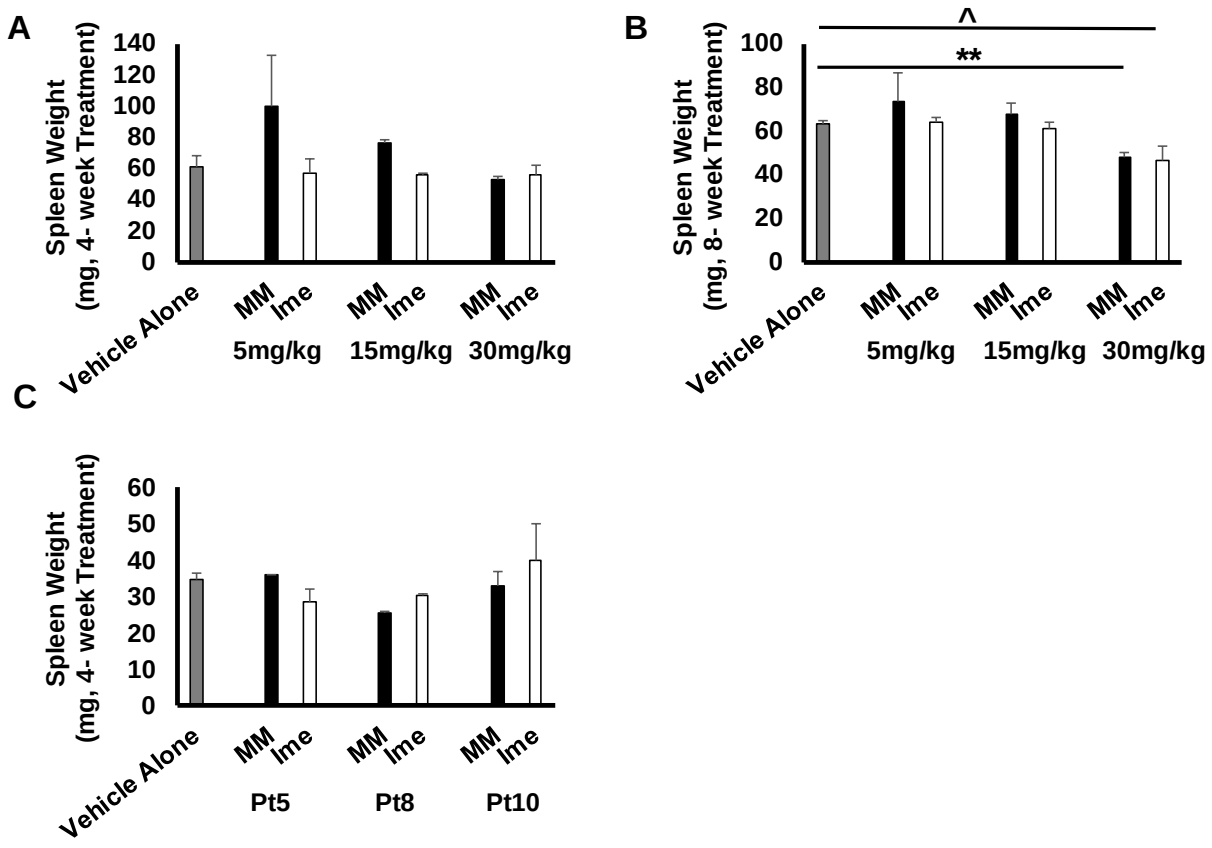

Figure S5: Spleen weight of NSG mice 4 months after the transplantation of normal CB or splenic MF CD34$^+$ cells. (A, B) NSG mice were transplanted with CB CD34$^+$ cells and were treated with vehicle alone, MM or imetelstat (5-30mg/kg) for 4 weeks (A) or 8 weeks (B) and their spleens were weighed 4 months after the transplantation (2-3 months after the discontinuation of the drug treatment). The treatment with imetelstat or MM for 4 weeks at each of the doses did not affect the weight of the mouse spleens. However, the prolonged treatment of these mice with higher doses of either imetelstat or MM (30mg/kg) resulted in a significant reduction in spleen weight as compared with vehicle alone treatment, respectively. Three samples of pooled CD34$^+$ cells from 8-10 CB donors were transplanted. ** $P<0.01$; ^ $P=0.056$. **(C)** NSG mice were transplanted with splenic CD34$^+$ cells from 3 patients with MF and were treated with vehicle alone, MM or imetelstat (15 mg/kg) for 4 weeks. Treatment with imetelstat resulted in a reduction in the spleen weight of the mice receiving splenic CD34$^+$ cells from Pt5 by 18.6% and 20.8% as compared with treatment with vehicle alone and MM respectively. The spleen sizes didn't change in mice transplanted with splenic CD34$^+$ cell from the other 2 patients following the treatment with either imetelstat or MM.

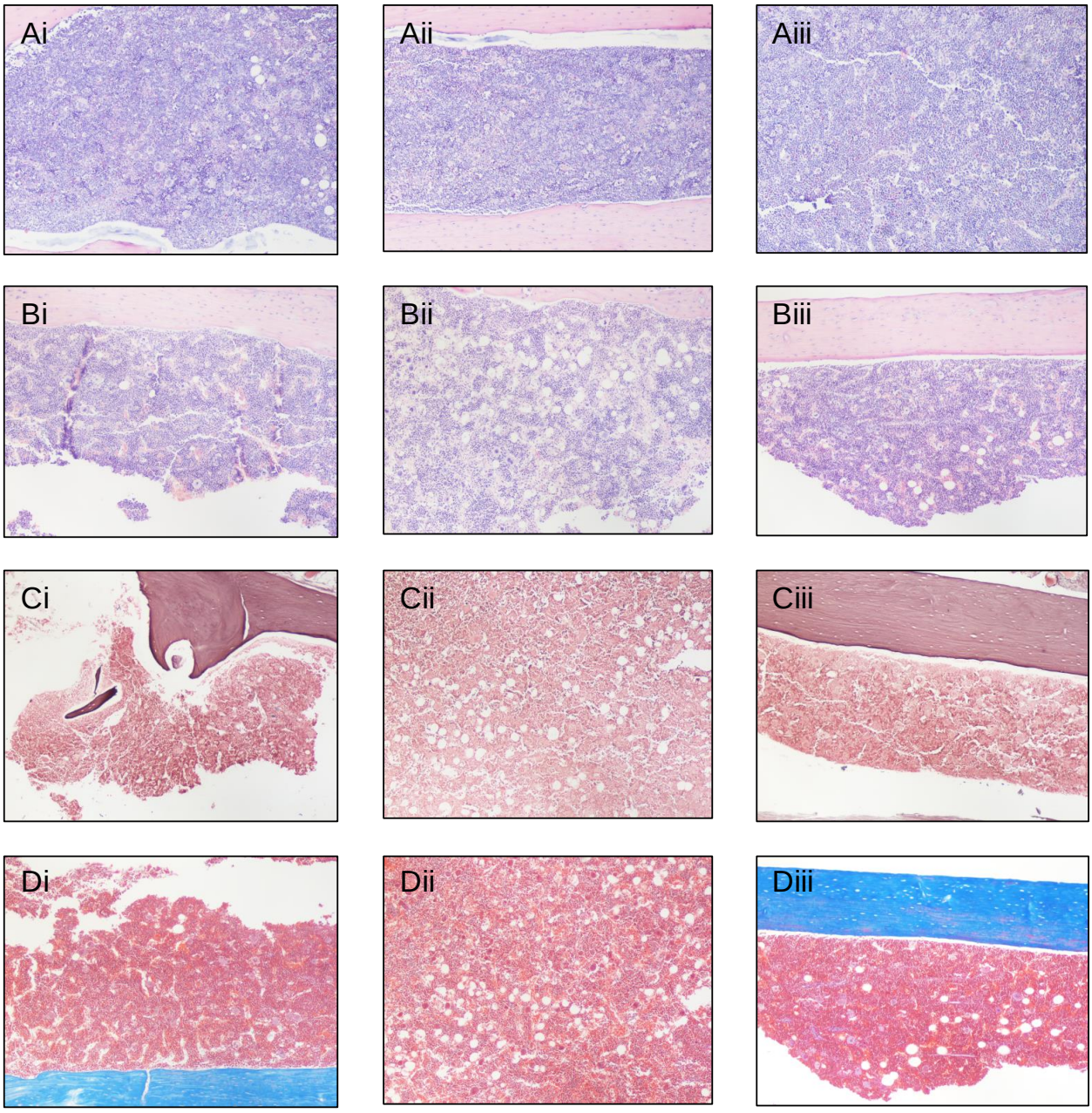

Figure S6: Histological analyses of normal or MF xenograft mice. (A, B) Cellularity assessment of the bone marrow (BM) of NSG mice transplanted with CB (A) or MF splenic (B) $CD34^+$ cells and treated with vehicle alone (i), MM (ii) or imetelstat (iii, 15mg/kg) for 4 weeks. H&E staining showed that the BM of mice transplanted with CB $CD34^+$ cells had similar 90-100% cellularity under each of the drug treatment. The BM of mice transplanted with MF splenic $CD34^+$ cells and treated with vehicle alone or imetelstat also had 90-100% cellularity (i, iii). The treatment with MM (ii), however, slightly reduced the BM cellularity to about 80%. **(C, D)** Fibrosis assessment of the bone marrow of NSG mice transplanted with MF splenic $CD34^+$ cells and treated with vehicle alone (i), MM (ii) or imetelstat (iii, 15mg/kg) for 4 weeks. C: Reticulin staining; D: Trichrome staining. No fibrosis was observed in any of these specimens. Representative results from the mice transplanted with $CD34^+$ cells of pooled CB2 $CD34^+$ cells and Pt5 splenic $CD34^+$ cells are shown.